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Relative quantification of genomic DNA fragments extracted from a biological tissue

Final report of the CCQM-P113

P. Corbisier

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European Commission
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Institute for Reference Materials and Measurements

Contact information
Address: Retieseweg 111, B2440 Geel, Belgium
E-mail: philippe.corbisier@ec.europa.eu
Tel.: +32 14 571 890
Fax: +32 14 571 548

http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

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2 Introduction

The provision of traceable standards to the biological community is an area of active research in many NMIs. The quantification of the relative amount of DNA sequences extracted from a biological tissue remains a complex analytical procedure and relies on the availability of such standards. Real-time PCR is currently the most applied measurement method to identify and quantify DNA sequences. Several NMIs were able to demonstrate their ability to use this technology to quantify a defined plasmid DNA using the same plasmid DNA as a calibrant (CCQM-P44 (1&2) and CCQM KC-61). The same measurement method was used to quantify genomic DNA extracted from a plant tissue and calibrated by a genomic DNA solution extracted from the same plant material (CCQM-P60). In a later study, the importance of a reliable DNA extraction method became apparent. The analytical procedure was more complex in CCQM-P60 compared to KC61, as it included a DNA extraction step. However, both studies were performed using matching calibrants for which a reference value had been assigned.
The goal of this pilot study was to demonstrate the ability to quantify DNA sequences present in a biological tissue using an independent calibration system. The quantification should ideally be performed by quantitative real-time PCR (QRT-PCR), but other methods not relying on the efficiency of thermal amplification such as digital PCR could also be applied.
The methodology requires extraction and purification of genomic DNA and accurate detection and quantification of the relative amount of two defined DNA sequences in the extracted genomic DNA.

3 Description of the Measurands

Both samples to be analysed are maize powders each containing a low but different number of copies of the same defined DNA sequence. The estimated number of copies to be detected ranged from 20 to 2 000 00 copies.

The ratio between the number of those two DNA sequences in the sample 1 and 2 must be determined:

Target sequence 1: (58 bp)
CTTTgCCAAgATCAAgCggAgTgAgggCCTTgAgTTAggCCATTCTggCCgAAgACTA

Target sequence 2: (79 bp)
GCTACATAgggAgCCTTgTCCTACAATCCACACAAACgCACgCgTAAAACAATTAAT
CAgCACgAgATTTCTAgTCCAA

The primer and probe sequences to be used for real-time PCR amplification are given in Annex 3.

4 Samples to be analysed

4.1 Description

Samples 1 and 3 were ERM-BF418b[1] whereas samples 2 and 4 were ERM-BF418c[2]. Both CRMs are maize powders each containing a defined mass fraction of genetically modified (GM) 1507 maize[3]. The Certified Reference Materials (CRMs) were produced and certified under the responsibility of the IRMM and were prepared by mixing of dried non-GM maize powder and 1507 GM dried maize powder subsequently. The mass fraction was certified taking the respective purity and water mass fractions into account.

4.2 Copy number ratio

Samples 2 and 4 were additionally certified to contain a defined ratio of the two DNA sequences present in 1507 maize. The ERM®-BF418c contains 0.49 ± 0.14 % 1507 copies per *hmg* copy. The certification is, however, on-going and the certified value not yet assigned.

Sample 1: was prepared gravimetrically and contains $\frac{1}{9.9}$ of the mass fraction of sample 2

Sample 2 : 0.49 ± 0.14 1507 copies/hmg copies [%]

Sample 3 : is identical to sample 1

Sample 4 : is identical to sample 2

4.3 Stability

The uncertainty contribution from the long-term stability was estimated by calculating the uncertainty on the mass fraction ratio of samples stored at 4 ºC and -70 ºC ($\bar{x}_{4\ ºC}/\bar{x}_{-70\ ºC}$), with -70 ºC being the reference temperature at which the material is considered to be stable. Accepting a shelf life of 18 months before additional stability measurements are required, a standard uncertainty contribution for the stability (u_{lts}) of 0.054 % was calculated. It is recommended to store the bottles at 4 ºC.

[1] http://www.irmm.jrc.be/html/reference_materials_catalogue/catalogue/attachements/ERM-BF418b_cert.pdf
[2] http://www.irmm.jrc.be/html/reference_materials_catalogue/catalogue/attachements/ERM-BF418c_cert.pdf
[3] Also named Herculex I TC1507 corn

5 Calibrant used

5.1 *Description*

The calibrant to be used was a candidate CRM (ERM-AD418). IRMM has constructed this plasmid DNA (pIRMM-0069) and has investigated its suitability for the calibration of quantitative measurements of maize event 1507. ERM-AD418 was processed according to the ISO Guide 34, certification is still on-going. The plasmid CRM is intended to be certified for the number of DNA fragments per plasmid of a 1507 transgenic sequence and of the high mobility group gene (*hmg*). The plasmid contains a 58 bp fragment of the transition region of the 35S-terminator into the ORF25 PolyA terminator and a 351 bp fragment of the maize endogenous *hmg* gene corresponding to the transition of the intron 4 into the exon 5 of the gene encoding the high mobility group protein A from *Zea mays*.

ERM-AD418 was prepared by diluting the pIRMM-0069 in a background of ColE1 plasmid DNA at a final concentration of 1 ng/µL. The buffer used for the dilution contained 1 mmol/L Tris, 0.01 mmol/L EDTA pH 8.0.

After its release ERM-AD418 is intended to be used for the calibration of 1507 maize QRT-PCR method as described and validated by the Community Reference Laboratory for GM Food and Feed[4].

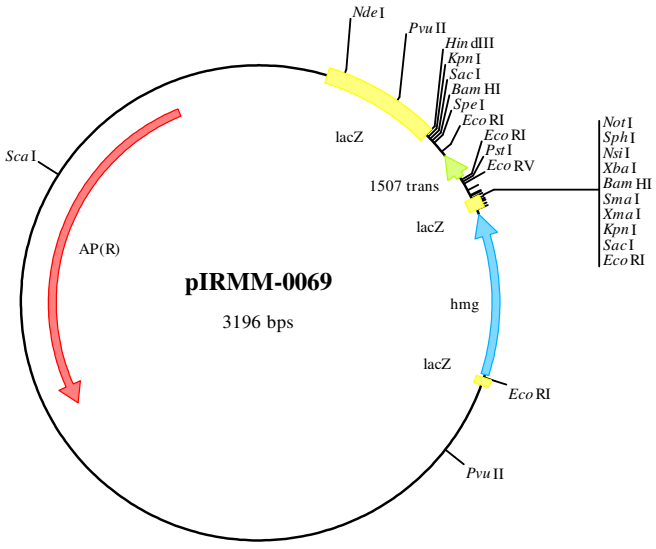


Figure 1: Circular map of pIRMM-0069 representing the 3'plant- junction and *hmg* inserts as well as the enzymatic restriction sites.

[4] http://gmo-crl.jrc.it/summaries/TC1507-WEB-Protocol-Validation.pdf

5.2 Purity of the calibrant

The purity of the plasmid pIRMM-0069 was analysed by agarose gel electrophoresis and no other DNA bands than those expected after restriction of the pIRMM-0069 with *Eco*RI (**Fig 2.** lane 2) and *Hin*dIII/*Xba*I (**Fig 2.** lane 3) could be seen after ethidium bromide staining. This confirmed the correct cloning of the fragments.

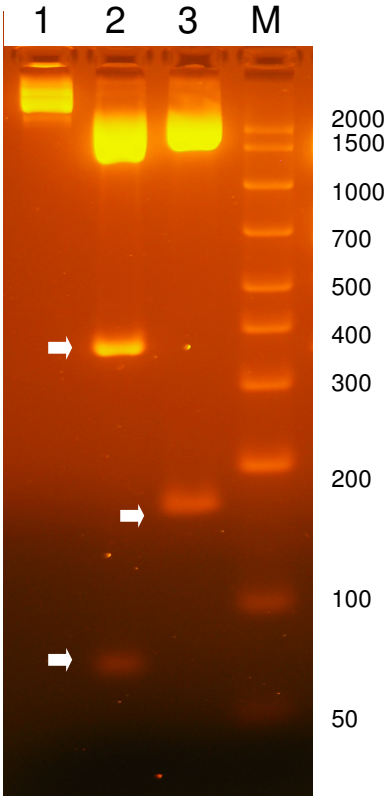


Figure 2: Restriction analysis of pIRMM-0069
Lane 1: pIRMM-0069 uncut; Lane 2: restricted with *Eco*RI (expected fragments: 2683 bp, 367 bp, 72bp, 74 bp); Lane 3: ERM-AD418 restricted with *Hin*dIII/*Xba*I (expected fragments: 3028 bp, 168 bp); M: molecular DNA marker (BioRad 50-2000 bp). White horizontal arrows indicate the positions of smaller fragments. The fragments sized 72 and 74 bp could not be discriminated under the electrophoresis conditions applied here.

As no smear was visible after restriction with *Eco*RI restriction enzyme of the plasmid preparation and no RNA band was visible, it can be reasonably concluded that the plasmid preparation was not contaminated with external genomic DNA or a relatively large amount of RNA molecules. However, traces of genomic DNA from host bacterial cell or traces of RNA molecules can not be excluded in the final plasmid preparation.
Such traces do not influence the target sequence ratio. Indeed a BLASTN 2.2.18 analysis of the cloned target sequences did not reveal any nucleic acid sequences identity with bacterial genomic DNA sources from the NCBI databases (data not shown).

Additionally, remaining traces of *E. coli* genomic DNA or RNA would not affect the measurement as the primers and probe used are highly specific for the targeted sequence and do not hybridise to other DNA fragments that could be present in the final preparation. Such traces may represent, however, a bias in the DNA quantification of the plasmid solution by UV and therefore an erroneous estimation of the number of plasmid copies in the tube. For that reason the DNA concentration in each tube can only be given as an approximate value.

Despite the fact that the enzymatic restriction conditions were chosen to allow a full digestion of the intermediated plasmids used for the assembly of pIRMM-0069, it is very difficult to prove that all plasmid populations were indeed fully digested, as traces of undigested plasmids will not be visible after gel electrophoresis and EtBr staining.

The *E. coli* cells could, consequently be transformed with 3 populations of plasmids: pIRMM-0069 present in large amount and traces of both undigested pIRMM-0067 and pIRMM-0068. However, as those 3 synthetic plasmids have the same origin of replication (*ori*V from ColE1 plasmid) they belong to the same incompatibility group. As a result, the transformed bacterial clones can only bear one single plasmid. As the plasmid production was intended to be started from a unique colony, only one type of plasmid can be present in a single colony. We could therefore conclude that each single bacterium extracted from one colony contains only one type of plasmid[5].

As additional proof of purity, plasmid DNA isolated from the transformed *E. coli* cells was sequenced completely to verify that both target DNAs were present and correctly cloned. The sequence analysis did not reveal the presence of a mixed population of plasmids.

Participants had the possibility to use any other type of calibrant which they think enables them to report the copy number ratio of 1507 and *hmg* fragments.

5.3 Homogeneity testing

Homogeneity analysis of the DNA sequences was performed at IRMM, where 19 vials were analysed using a QRT-PCR method. The relative standard deviation was found to be less than 12 %. The sample intake used for determining the homogeneity was 200 mg.

[5] The synthetic vectors used (pUC18 and pCR®2.1) in our cloning strategy were chosen as being high copy vectors from the same incompatibility group (incQ). Those plasmids have their own origin of replication (*oriV*) and are able to replicate independently of the host chromosome. A bacterial host cannot however contain different plasmids that have the same mechanisms of replication, because the control of the replication (in Gram negative bacteria) is exercised through trans-acting molecules (theta replication mode). The inevitable consequence of this is that one of the plasmids would eventually be lost from the cell simply as a result of random partitioning of plasmids into daughter cells during cell division. Thus the plasmids would appear to be incompatible. Two or several plasmids from the same incompatibility group cannot coexist in the same cell.

5.4 Stability testing

As the 1507 plasmid calibrant ERM-AD418 has been produced following the same procedure as the ERM-AD413 produced in November 2006 and is of the same nature, long term-stability study for the ERM-AD418 was replaced by a post-certification study of the stability of ERM-AD413 for a period of 12 and 24 months. Tubes containing the ERM-AD413 have been stored to -20 °C and 18 °C to be analysed at those respective time. The tubes stored for a period of 12 months were analysed (**Fig. 3**). The data for the 24 months will only be available in November 2008. On the basis of the 12 months stability study, a shelf-live of 24 months can be guaranteed with a relative uncertainty of 8.4 % and 8.5 % for the material stored at 18°C or -20°C, respectively.

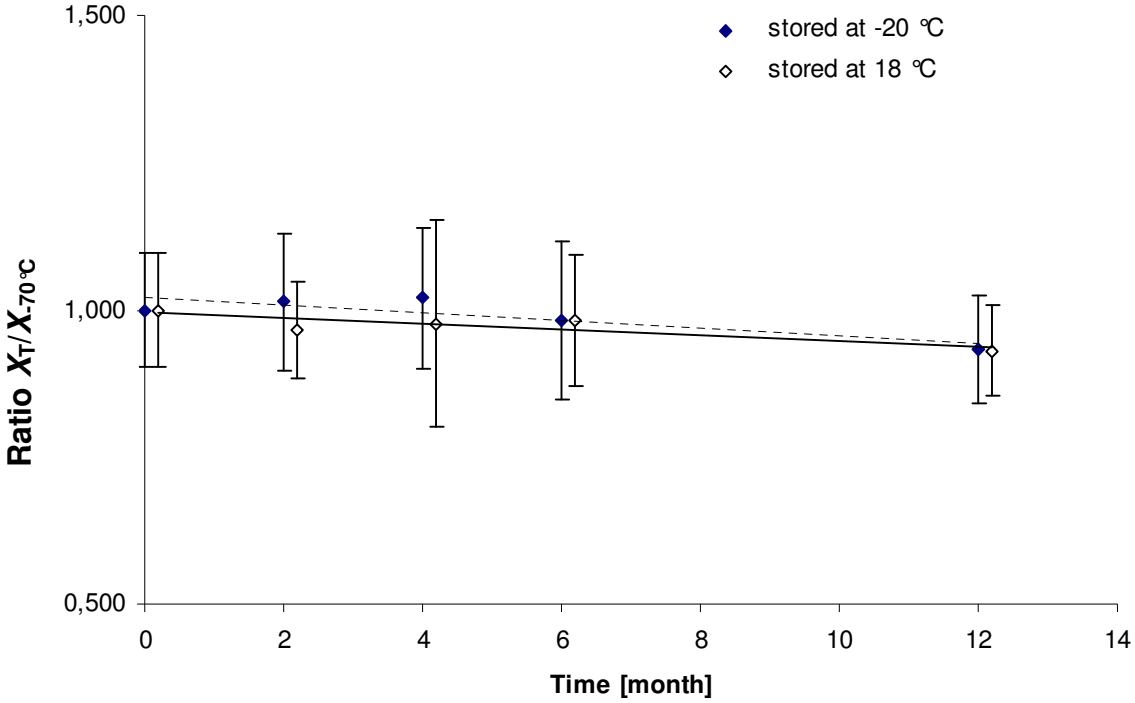


Figure 3: Long-term stability of plasmid DNA (ERM-AD413, MON 810 calibrant) stored at -20 °C and 18 °C for 12 months based on rt-PCR measurements. The stability is expressed as the ratio between the number ratio of MON 810 and *hmg* fragments per plasmid in samples stored at -20 °C or 18 °C and that in sample stored for the same period at the reference temperature (-70 °C), with the bars indicating the expanded uncertainty interval ± U (k = 2). Each bullet corresponds to the average of 5 to 11 measurements. The dashed line is the regression line generated on the basis of the -20 °C data points, whereas the full line is the regression line generated on the basis of the 18 °C data points.

Based on the 12 month stability study of ERM-AD413, a minimum shelf live of 24 months at -20 °C can be guaranteed with a relative uncertainty (u_{lts}) of 8.5 %. As the number ratio stays unchanged for tubes stored at -20 °C or at 18 °C, the material will not suffer from storage at 4°C. The apparent trend visible in Figure 3 proves to be not statistically significant at a confidence level of 95%.

5.5 Instructions for use

Participants have received four glass bottles. Two of those bottles contained 1 g of ERM-BF418b and 2 bottles contained ERM-BF418c. Those samples should be stored at 4 ºC. A tube containing the candidate ERM-AD418 calibrant should be stored at -20 ºC.
Participants were requested to analyse the 4 samples on 2 different days:

Day 1

1. Three independent DNA extractions from sample 1 (U1 to U3) and sample 2 (U4 to U6) containing an unknown amount of copies of the 1507 event
2. DNA quantification of U1 to U3 and of U4 to U6
3. Preparation of dilutions of the plasmid DNA or any other suitable calibrant
4. Real-time PCR (or other methods quantifying DNA fragments)

Day 2

On Day 2 steps 1 to 4 will be repeated performing three DNA extractions (U7 to U9) from sample 3 and (U10 to U12) for sample 4.
A detailed protocol on the use of the calibrant will be provided along with the samples. The general use of such calibrant is also described in the ERM Technical Note 5[6].

	DNA extracted from:		Preparation of dilution series from:
Day 1	Sample 1	U1, U2, U3	Calibrant 1 or any other suitable calibrant
	Sample 2	U4, U5, U6	
Day 2	Sample 3	U7, U8, U9	New dilution of calibrant 1 or any other suitable calibrant
	Sample 4	U10, U11, U12	

5.6 Methodology

Participants were requested to use their preferred methodology for the extraction of genomic DNA from the samples. Special care should be taken to prepare highly purified genomic DNA. The unknowns could be diluted to verify the absence of PCR inhibitors in the extracted DNA. The extraction method referred to by the CRL for GM Food and Feed can for example be applied[7].
QRT-PCR is the most commonly used method to quantify DNA sequences but other technologies can also be used. The PCR platform and chemistry could be chosen by the participants. One laboratory performed digital PCR on a Biomark™ system with digital arrays.

[6] http://www.erm-crm.org/html/ERM_products/application_notes/application_note_5/application_note_5_english.pdf

[7] http://gmo-crl.jrc.it/summaries/TC1507-DNAextrc.pdf

6 Participants

The 14 NMIs or designated laboratories were originating from 4 continents and distributed between the Asian-Pacific (8 participants) and North American (3 participants) regions and Europe (3 participants). The Asian-pacific participation was strongly represented and provided more than half of the total number of data sets.

Table 1: List of the participants to the CCQM P113 pilot study and contact person. * Organising laboratory.

Thailand	NIM- Thailand	Duangkamol Viroonudomphol
	DMSC-Thailand	Nittaya Phunbua
Rep of China	NMI PR China	Yunhua Gao
Hong Kong	Hong Kong Government Lab	Chun-yin Mak
Japan	NFRI	Satoshi Furui
	NMI	Mamoru Kawaharasaki
Rep of Korea	KRISS	Hyong-Ha Kim
Australia	NMIA	Kerry Emslie
Canada	NIM	Mike Rott
USA	NIST	Marcia Holden
Mexico	CENAM	Melina Pérez Urquiza
United Kingdom	LGC	Malcolm Burns
European Commission	IRMM*	Philippe Corbisier
Russian Federation	VNIIM	Maxim Vonsky

7 Timing

The laboratories had to signup for the pilot study before 1st of February 2008, the material was sent in the week of 24th March 2008 and results had to be submitted before the 6th of June 2008. A few results came later but were still accepted for the study. This draft report has been sent on 1st of November 2008 to be discussed at the Bioanalysis Group Meeting in Bangkok on 18th November 2008.

8 Results

The results reported here are those reported for the sample 1 to 4 and expressed in percentage of 1507 copies per *hmg* copies. Each laboratory has received a random identification number. Affiliations are not disclosed.

The copies number percentage for samples 1 and 3 were chosen to be at 1/20 of the level of the European labelling threshold for food and feed. It represents a very low number of 1507 copies in the sample (about 10 copies of 1507 targets per PCR). Several laboratories have reported the measured value as being under the practical limit of detection for the subsequent dilutions. In a few cases, the measured Ct values were outside the calibration curves. In this report, the GM percentages have been calculated on basis of the undiluted samples to have an idea about the performance of the QRT-PCR at very low copy number.

9 Extraction method applied

The DNA extraction method that was applied to extract the genomic DNA from the samples was left to the discretion of the laboratories and are summarised in Figure 1 and 2.

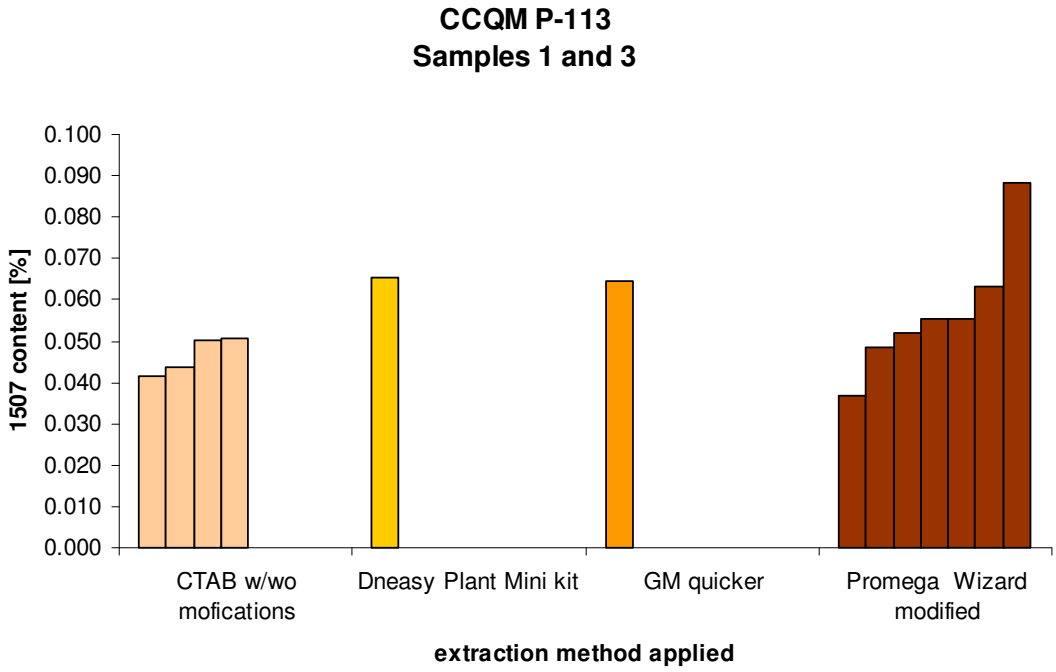


Fig 1: DNA extraction method applied on samples 1 and 3.

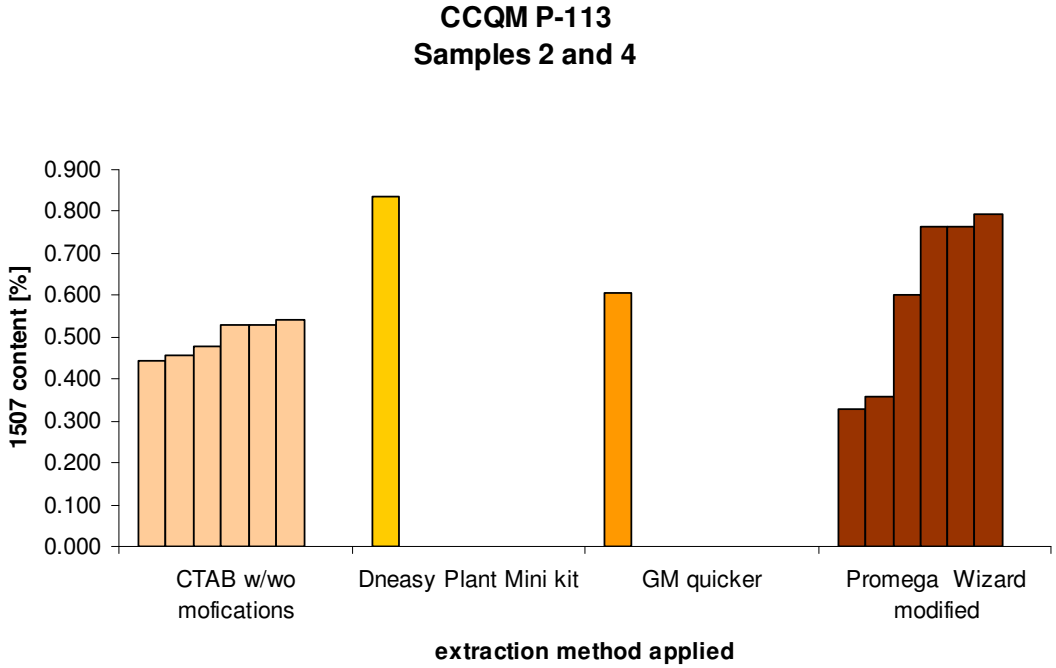


Fig 2: DNA extraction method applied on samples 2 and 4.

The Dneasy and GM quicker methods were each applied by one single laboratory, whereas the other laboratories performed either a CTAB method with for some an additional final purification step on a column or applied the Promega Wizard extraction protocol which is using property binding columns from which DNA is eluted. There is no clear indication that one extraction method provides better quantification results than another. However a statistical analysis still needs to be performed.

10 Results

10.1 Sample 1

The sample 1 has been quantified by real-time PCR. Lab2b did an additional absolute quantification of the respective number of 1507 and *hmg* copies by digital PCR. The value reported by Lab2b is in very good agreement with the expected reference value.
The labs 9 and 10 are outliers and data should be confirmed by those laboratories. The value for lab 10 is based on an average of sample 1 and 3 as individual data points could not be retrieving. The PCR efficiencies for Lab 10 were very low suggesting some serious problem with the measurement.

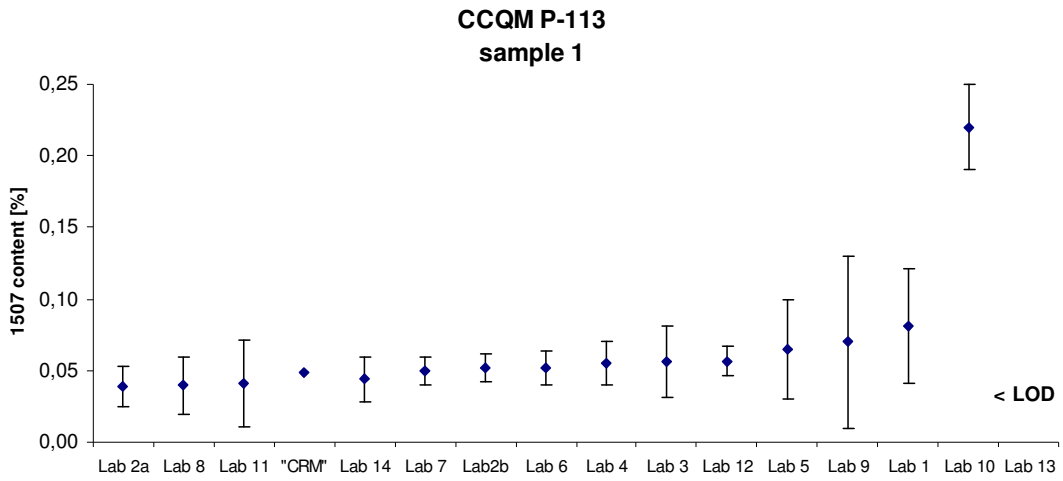


Fig 3: Distribution of the average GM content measured on sample 1. Error bars represented the expanded uncertainty ($k = 2$). Lab 13 reported the value as being below their LOD.

The "CRM" value has been extrapolated as being 1/9.9 of the currently estimated value of 0.49 % ± 0.14 as samples 1 and 3 contains 9.9 times less 1507 mass than sample 2 and 4. The CRM is bracketed as the certificate has not yet been released.

10.2 Sample 3

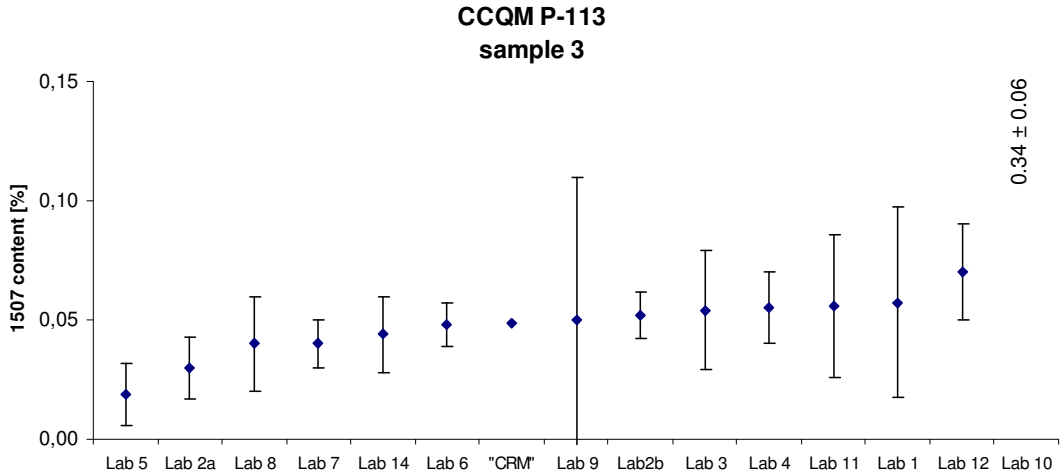


Fig 4: Distribution of the average GM content measured on sample 3. Error bars represented the expanded uncertainty ($k = 2$).

The sample 3 was the same as sample 1 but the DNA was extracted and analysed on another day. The results are similar to those observed for sample 1.

10.3 Sample 2

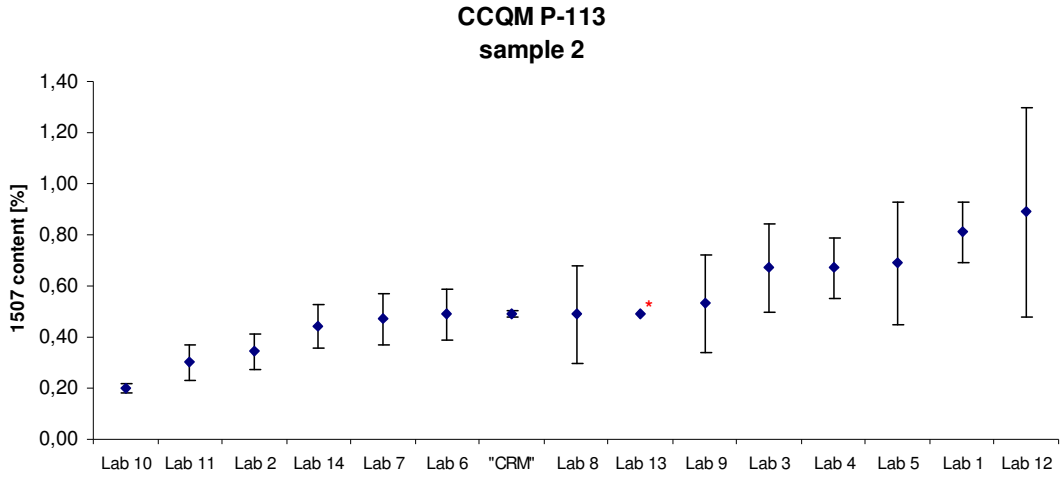


Fig 5: Distribution of the average GM content measured on sample 2. Error bars represented the expanded uncertainty ($k = 2$). * no uncertainty reported.

The sample 2 has been analysed by 35 laboratories in an inter comparison trial. The candidate CRM is estimated to have a 1507 copy number ratio of 0.49 ± 0.14 %. That value is also reported by several NMIs, however the data points are fluctuating between 0.35 and 1 %.

10.4 Sample 4

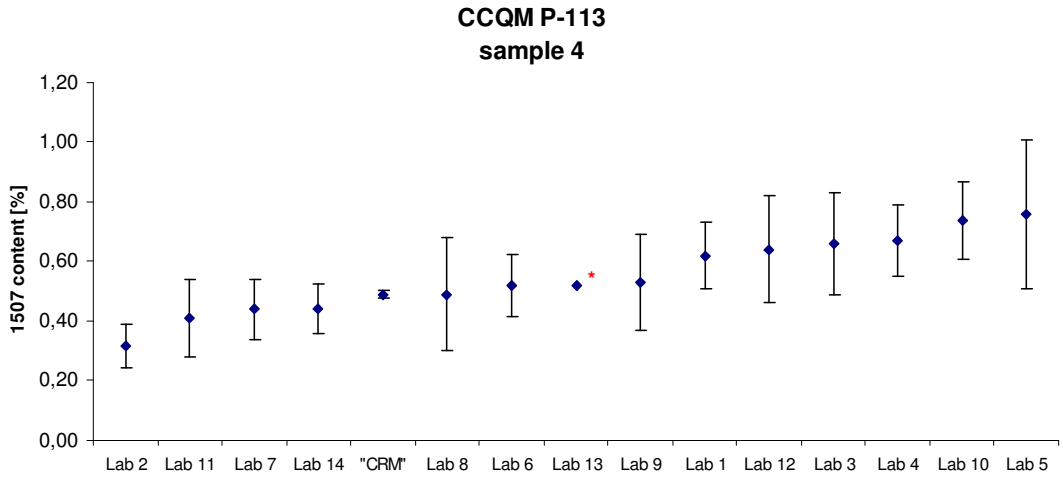


Fig 6: Distribution of the average GM content measured on sample 1. Error bars represented the expanded uncertainty ($k = 2$). * no uncertainty reported.

The sample 4 was identical as sample 2 but the DNA was extracted and analysed on another day.

10.5 Combined samples 1 and 3

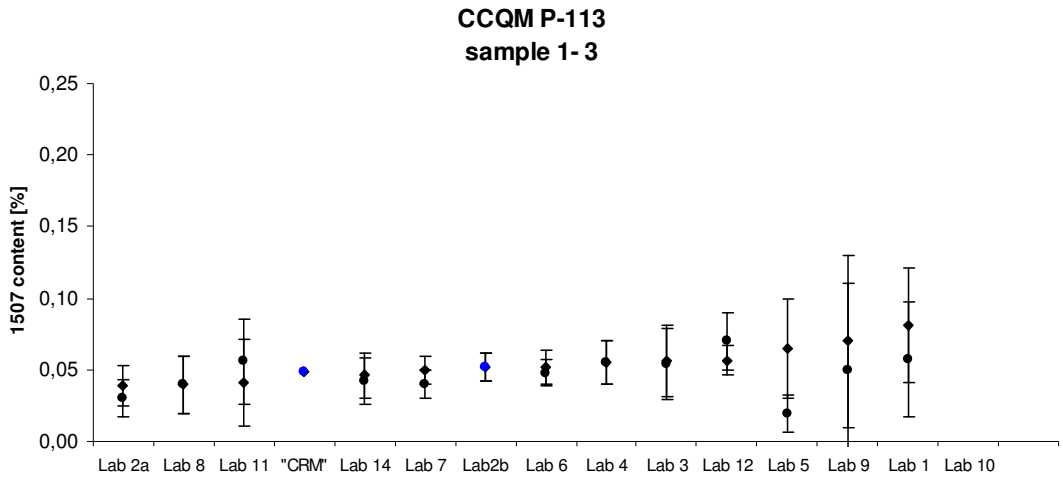


Fig 7: Distribution of the average GM content measured on samples 1 and 3. Error bars represented the expanded uncertainty ($k = 2$).

Most laboratories do have a very good repeatability of their measurement at this very low number of 1507 target DNA copies.

10.6 Combined samples 2 and 4

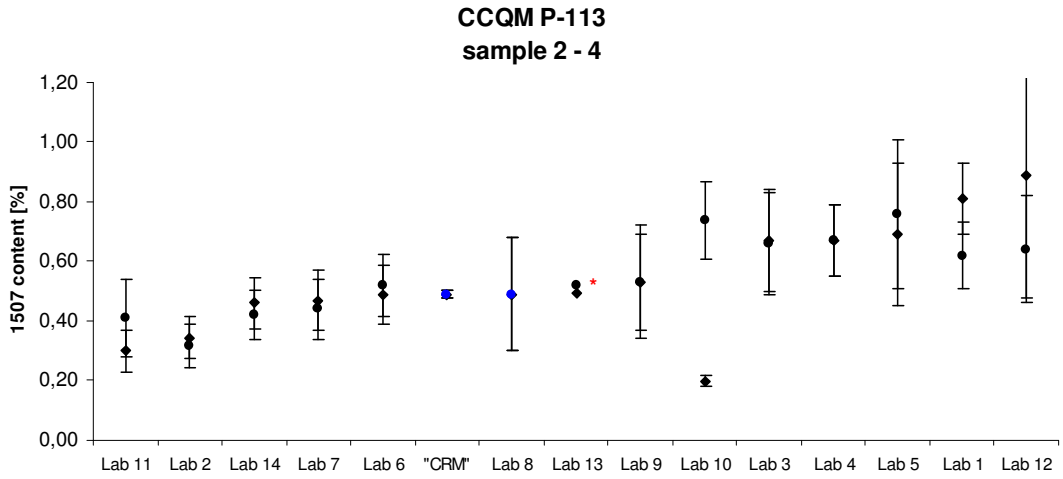


Fig 8: Distribution of the average GM content measured on samples 2 and 4. Error bars represented the expanded uncertainty ($k = 2$). * no uncertainty reported.

Despite the higher concentration of 1507 targets in samples 2 and 4 compared to 1 and 3, the repeatability does not seem to be improved for several laboratories.

11 Summary table

Table 2 provides a summary of the average GM quantification reported in the 4 samples together with the calculated expanded uncertainty (k=2).

	sample 1		**sample 3**		**sample 2**		**sample 4**	
	mean	*U*	mean	*U*	mean	*U*	mean	*U*
Lab 1	0.08	± 0.04	<LOD		0.81	± 0.12	0.62	± 0.11
Lab 2a	0.039	± 0.014	0.03	± 0.013	0.342	± 0.07	0.321	± 0.073
Lab 2b	0.052	± 0.010						
Lab 3	0.055	± 0.025	0.055	± 0.025	0.67	± 0.17	0.66	± 0.17
Lab 4		0.055 ± 0.015				0.67 ± 0.12		
Lab 5	0.065	± 0.035	0.019	± 0.013	0.69	± 0.24	0.76	± 0.25
Lab 6	0.052	± 0.012	0.048	± 0.009	0.49	± 0.0996	0.52	± 0.1037
Lab 7	0.05	± 0.02	0.04	± 0.02	0.4	± 0.1	0.5	± 0.1
Lab 8		0.04 ± 0.02				0.49 ± 0.19		
Lab 9	0.07	± 0.06	0.05	± 0.06	0.53	± 0.19	0.53	± 0.16
Lab 10	0.22	± 0.03	0.34	± 0.06	0.20	± 0.02	0.74	± 0.13
Lab 11	0.041	± 0.03	0.06	± 0.03	0.301	± 0.07	0.41	± 0.13
Lab 12	0.056	± 0.01	0.07	± 0.02	0.89	± 0.41	0.64	± 0.18
Lab 13					0.492		0.521	
Lab 14		0.044 ± 0.14				0.442 ± 0.085		
All Labs	0.06		0.05		0.56		0.53	
"target" value sample 2/4					0.49	± 0.21		
CCQM 113	0.05	± 0.01			0.55	± 0.06		

12 Conclusions

The goal of this CCQM 113 pilot study was to demonstrate the ability to quantify DNA sequences present in a biological tissue using an independent calibration system. The quantification has been performed by QRT-PCR. One laboratory has performed an absolute quantification of both targets by digital PCR without the use of an external standard on sample 1 and found precisely the expected GM concentration. This result reveals the potential of such new technology for absolute quantification of DNA targets.

The samples 1 and 3 had a very low number of 1507 targets which has created some difficulties for several laboratories as the unknown samples were too diluted and falling outside calibration curves. The data for the undiluted samples have nevertheless been reported here.

Most participating NMIs managed to quantify the 4 samples with high accuracy and reported measurements very close to the assigned value. They could clearly demonstrate their ability to quantify DNA sequences in a biological tissue.

The assigned value of sample 2 and 4 is based on inter laboratory comparison including 35 data sets. The list of those participating laboratories is given in Annex 1.

The expanded uncertainty associated to that material comprises standard uncertainty contributions from the characterisation, the heterogeneity, and the stability and is reported in Annex 2.

The expanded uncertainties (k = 2) related to those measurements have been calculated in different ways and are varying a lot between the laboratories. The uncertainties have been determined either by the bottom-up or top-down approaches. Details received from the participating laboratories are given in Annex 5.

13 Contributors to the study

CENAM: Melina Perez Urquiza**,** Martha Graciela Rocha Munive (INE-CENAM)
DMSC Thailand: Nittaya Phunbua
Hong Kong Government Laboratory: Chun-yin Mak
IRMM: Philippe Corbisier, Sandra Vincent, Heinz Schimmel
KRISS: Hyong-Ha Kim, Woo Jeong Kim
LGC: Carole Foy, Steve Ellison, Malcolm Burns
NFRI: Satoshi Furui
NIST: Marcia Holden, Marc Salit, Ross Haynes
NMI Canada: Mike Rott
NMI China: Gao Yunhua
NMI Japan: Mamoru Kawaharasaki
NMI Thailand: Duangkamol Viroonudomphol
NMIA: Kerry Emslie, Lina Partis, Somanath Bhat, Thosaporn Coldham, Daniel Burke, Vicki Rui Dan Xie and Gusharan Bains
VNIIM: Maxim Vonsky

14 Annex 1 : Laboratories involved in the certification of the samples 2 and 4 (ERM-AD418c)

-Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Dienststelle Oberschleißheim, DE* (Staatliche Anerkennungsstelle der Lebensmittelüberwachung, SAL-BY-L20-04-03)
-Crop Research Institute, Prague 6 – Ruzyne, CZ* (Czech accreditation institute, nº8/2007)
-Danish Plant Directorate, Laboratory for Diagnoses in Plants, Food and Feed Lyngby, DK* (DANAK 330)
-Ente Nazionale Delle Sementi Elette (ENSE), Laboratorio Analisi Sementi, Tavazzano, IT* (ISTA IT03)
-Eurofins Analytik GmbH - WEJ Dept Biology 135, Hamburg, DE* (Deutsches Akkreditierungssystem Prüfwesen GmbH, DAP-PL-1453.00)
-European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM) - RM Unit, Geel, BE* (BELAC, 268-test)
-Finnish Customs Laboratory - Tullilaboratorio, Espoo, FI* (FINAS, T006)
-Groupe d'études et de contrôle des variétés et des semences (GEVES) - BioGEVES, Surgères, FR* (COFRAC, 1-1540)
-Hainaut Vigilance Sanitaire - Institut Provincial d'Information et d'Analyses Sanitaires, Mons, BE* (Beltest, M-089T)
-Federale Overheidsdienst (FOD) Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu Wetenschappelijk Instituut Volksgezondheid, Afdeling Bioveiligheid en Biotechnologie, Brussel, BE* (BELAC, 081-test)

-Institut für Hygiene und Umwelt – Behörde für Umwelt und Gesundheit, Hamburg, DE* (Deutsches Akkreditierungsstelle Chemie GmbH, DAC-PL-0137-01-10)
-Instituut voor Landbouw- en Visserijonderzoek (ILVO), Melle, BE* (BELAC, 033-test)
-Korea Research Institute of Standards and Science (KRISS) - Organic and Bio Analysis Group, Daejeon, KR
-Landesamt für Umweltschutz Sachsen-Anhalt, FG 13 Gentechnisches Überwachungslabor, Halle/Saale, DE* (AKS, AKS-PL-21505)
-Lifeprint GmbH, Illertissen, DE* (Deutsches Akkreditierungssystem Prüfwesen GmbH, DAP-PL-3515.00)
-Livsmedelsverket - National Food Administration – Biology Division, Uppsala, SE* (SWEDAC, 1457)
-National Food Research Institute (NFRI) - Molecular Engineering Lab, Tsukuba, JP* (International Accreditation Japan, ASNITE 0018R)
-National Institute for Food Safety and Nutrition, Budapest, HU* (NAT, NAT-1-1161/2003)
-National Institute of Biology (NIB), Ljubljana, SI* (Slovenska akreditacija, LP-028)
-National Veterinary Laboratory of the Republic of Lithuania, Vilnius, LT* (Deutsches Akkreditierungssystem Prüfwesen GmbH, DAP-PL-3328.99)
-Nestlé Research Center Lausanne (Nestec S.A.) - Department Quality & Safety Assurance, Lausanne, CH* (Swiss Accreditation Service, STS 188)
-New Technical University of Denmark - National Food Institute, Søborg, DK* (DANAK, 350)
-Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), Lebensmittelinstitut Braunschweig, Braunschweig, DE* (Staatliche Akkreditierungsstelle Hannover, AKS-PL-10301)
-Ontario Plant Laboratories - Canadian Food Inspection Agency - Ottawa Laboratory Fallowfield, Ottawa, CA* (Standards Council of Canada, 316)
-Scottish Agricultural Science Agency - Diagnostics & Molecular Biology Section, Edinburgh, UK
-Staatliches Gewerbeaufsichtsamt Hildesheim - Dez 33 Gentechnik, Hildesheim, DE* (Deutsches Akkreditierungsstelle Chemie GmbH, DACH/DAC-P-0257-04-00)
-TNO Nutrition and Food Research - GMO foods, Zeist, NL* (Dutch accreditation council RvA L027)
-Umweltbundesamt Wien (UBA Wien), Vienna, AT* (Federal Ministry of Economics and labour, 200)
-USDA, Grain Inspection, Packers and Stockyards Administration - Technical Services Division, Kansas City, USA

15 Annex 2 : Uncertainty budget for the number fraction of maize event 1507 in the candidate calibrant ERM-AD418

The value is the number of each cloned DNA fragment per plasmid. The number ratio between those two DNA fragments is given as an indicative value measured by simplex real-time PCR.

substance	value [number]	Uncertainty
Fragment of 3'-insertion-specific DNA per plasmid pIRMM -0069 [a)]	1	negligible
Fragment of *hmg* DNA per plasmid pIRMM -0069 [1)]	1	negligible

	Indicative value [number ratio]	Uncertainty [c)] [number ratio]
Ratio between the numbers of 3'-insertion-specific DNA and *hmg* fragments in the plasmid pIRMM -0069	1.00 [b)]	0.14

[a)] The 100 % sequence identity has been confirmed by dye terminator cycle sequencing of the *hmg* and 3' insert-plant junction fragments both present in *Zea mays* DAS-Ø15Ø7-1.

[b)] The number ratio for the two DNA fragments has been characterised by simplex QRT-PCR targeting each cloned fragment.

[c)] The uncertainty is the expanded uncertainty estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) with a coverage factor k = 2, corresponding to a level of confidence of about 95 %.

16 Annex 3: Uncertainty budget for the DNA copy number ratio of 1507 maize in ERM-BF418c.

The expanded uncertainty of the value (U_{CRM}) comprises standard uncertainty contributions from the characterisation, the heterogeneity, and the stability.

$$U_{CRM} = k\sqrt{u_{\text{char}}^2 + u_{\text{bb}}^2 + u_{\text{lts}}^2}$$

with $k = 2$

The uncertainty from the characterisation has been assessed during the interlaboratory comparison by estimating the RSD of the normally distributed data. The standard uncertainty (u_{char}) related to the characterisation is calculated using the formula:

$$u_{\text{char}} = \frac{s}{\sqrt{N}}$$

Where s = standard deviation

N = number of data sets, in this study $N = 35$.

The uncertainty introduced by the heterogeneity at 200 mg level has been estimated on the basis of the heterogeneity of a normally distributed population. The uncertainty contribution from the stability (u_{lts}) has been estimated on the basis of QRT-PCR results following long-term storage of ERM-BF418c. A coverage factor of 2 ($k = 2$) was used to calculate the expanded uncertainty corresponding to a level of confidence of about 95 %.

The standard uncertainty contribution introduced by the long-term stability is larger than the standard uncertainty contributions from the characterisation and from the heterogeneity of the maize powders.
The value reported here is NOT the certified value as the material has not yet been released.

Value[8] **[%]**	**Standard uncertainty contributions [%]**			**Expanded uncertainty U_{CRM} (k = 2) [%]**
	u_{bb} a)	u_{lts} b)	u_{char} c)	U_{CRM}
0.49	0.041	0.054	0.016	0.14

a) Standard uncertainty introduced by the heterogeneity at 200 mg level.
b) Standard uncertainty related to the stability, estimated on the basis of a shelf life of 18 months.
c) Standard uncertainty introduced by the characterisation.

[8] Please note that as the material has not yet been released the value reported here is **NOT** the certified value.

17 Annex 3: Primer and probe sequences used for the quantification of the *hmg* and 1507 event-specific rt-PCR.

	Sequence (5' to 3')
1507-F primer (P-0487)	TAg TCT TCg gCC AgA ATg g
1507-R primer (P-0488)	CTT TgC CAA gAT CAA gCg
1507 probe (P-0489)	6-(FAM)-TAA CTC AAg gCC CTC ACT CCg-TAMRA-
hmg probe	6-(FAM)-CAA TCC ACA CAA ACg CAC gCg TA-TAMRA
hmg -F primer	TTg gAC TAg AAA TCT CgT gCT gA
hmg -R primer	gCT ACA TAg ggA gCC TTg TCC T

18 Annex 4 : Statistical analysis of the P-113 pilot study

Lab Alias	Abbreviation	Lab Full Name	Person Name / Telephon
L0	Lab1	not disclosed	not disclosed
L1	Lab2	not disclosed	not disclosed
L2	Lab3	not disclosed	not disclosed
L3	Lab4	not disclosed	not disclosed
L4	Lab5	not disclosed	not disclosed
L5	Lab6	not disclosed	not disclosed
L6	Lab7	not disclosed	not disclosed
L7	Lab8	not disclosed	not disclosed
L8	Lab9	not disclosed	not disclosed
L9	Lab11	not disclosed	not disclosed
L10	Lab12	not disclosed	not disclosed
L11	Lab13	not disclosed	not disclosed
L12	Lab14	not disclosed	not disclosed

Be aware that the Lab alias number in Annex 4 are different than the Lab number reported in the section 8. L0 corresponds to lab 1, L1 corresponds to Lab 2, etc...
The data of lab 10 have not been included as some clarification from Lab 10 is first needed.

Samples 2 and 4

Data from the Lab: L1

Mean of Lab ± StDev = 0,608 ± 0,052 [CV(%) = 8,6]

Sample's ID =>	Sample #2	Sample #4
Rep #1	0,53	0,495
Rep #2	0,698	0,636
Rep #3	0,706	0,581
Mean	0,645	0,571
STDev	0,099	0,071
CV(%)	15,416	12,453

Data from the Lab: L2

Mean of Lab ± StDev = 0,330 ± 0,019 [CV(%) = 5,7]

Sample's ID =>	Sample #2	Sample #4
Rep #1	0,36	0,29
Rep #2	0,36	0,29
Rep #3	0,31	0,37
Mean	0,343	0,317
STDev	0,029	0,046
CV(%)	8,408	14,586

Data from the Lab: L3

Mean of Lab ± StDev = 0,655 ± 0,006 [CV(%) = 0,9]

Sample's ID =>	Sample #2	Sample #4
Rep #1	0,638	0,658
Rep #2	0,721	0,766
Rep #3	0,594	0,555
Mean	0,651	0,660
STDev	0,064	0,106
CV(%)	9,906	15,994

Data from the Lab: L4

Mean of Lab ± StDev = 0,795 ± 0,064 [CV(%) = 8,1]

Sample's ID =>	Sample #2	Sample #4
Rep #1	0,821	0,775
Rep #2	0,848	0,734

Rep #3	0,853	0,74
Mean	0,841	0,750
STDev	0,017	0,022
CV(%)	2,048	2,954

Data from the Lab: L5

Mean of Lab ± StDev = 0,837 ± 0,203 [CV(%) = 24,2]

	Sample #2	Sample #4
Sample's ID =>		
Rep #1	1,4	0,55
Rep #2	0,7	0,91
Rep #3	0,84	0,62
Mean	0,980	0,693
STDev	0,370	0,191
CV(%)	37,796	27,530

Data from the Lab: L6

Mean of Lab ± StDev = 0,508 ± 0,025 [CV(%) = 4,9]

	Sample #2	Sample #4
Sample's ID =>		
Rep #1	0,436	0,621
Rep #2	0,556	0,473
Rep #3	0,478	0,482
Mean	0,490	0,525
STDev	0,061	0,083
CV(%)	12,427	15,794

Data from the Lab: L7

Mean of Lab ± StDev = 0,454 ± 0,015 [CV(%) = 3,3]

	Sample #2	Sample #4
Sample's ID =>		
Rep #1	0,476	0,462
Rep #2	0,485	0,463
Rep #3	0,434	0,406
Mean	0,465	0,444
STDev	0,027	0,033
CV(%)	5,854	7,353

Data from the Lab: L8

Mean of Lab ± StDev = 0,495 ± 0,016 [CV(%) = 3,2]

	Sample #2	Sample #4
Sample's ID =>		
Rep #1	0,603	0,514
Rep #2	0,485	0,443
Rep #3	0,43	0,493
Mean	0,506	0,483
STDev	0,088	0,036
CV(%)	17,469	7,546

Data from the Lab: L9

Mean of Lab ± StDev = 0,527 ± 0,005 [CV(%) = 0,9]

	Sample #2	Sample #4
Sample's ID =>		
Rep #1	0,52	0,53
Rep #2	0,56	0,53
Rep #3	0,49	0,53
Mean	0,523	0,530
STDev	0,035	0,000
CV(%)	6,711	0,000

Data from the Lab: L11

Mean of Lab ± StDev = 0,357 ± 0,079 [CV(%) = 22,2]

	Sample #2	Sample #4
Sample's ID =>		
Rep #1	0,34	0,563
Rep #2	0,253	0,308
Rep #3	0,31	0,369
Mean	0,301	0,413
STDev	0,044	0,133
CV(%)	14,682	32,215

Data from the Lab: L12

Mean of Lab ± StDev = 0,765 ± 0,177 [CV(%) = 23,1]

	Sample #2	Sample #4
Sample's ID =>		
Rep #1	1,21	0,49
Rep #2	0,86	0,68
Rep #3	0,6	0,75
Mean	0,890	0,640
STDev	0,306	0,135
CV(%)	34,394	21,021

Data from the Lab: L13

Mean of Lab ± StDev = 0,600 ± 0,009 [CV(%) = 1,6]

	Sample #2	Sample #4
Sample's ID =>		
Rep #1	0,59	0,59
Rep #2	0,57	0,64
Rep #3	0,62	0,59
Mean	0,593	0,607
STDev	0,025	0,029
CV(%)	4,241	4,758

Data from the Lab: L14

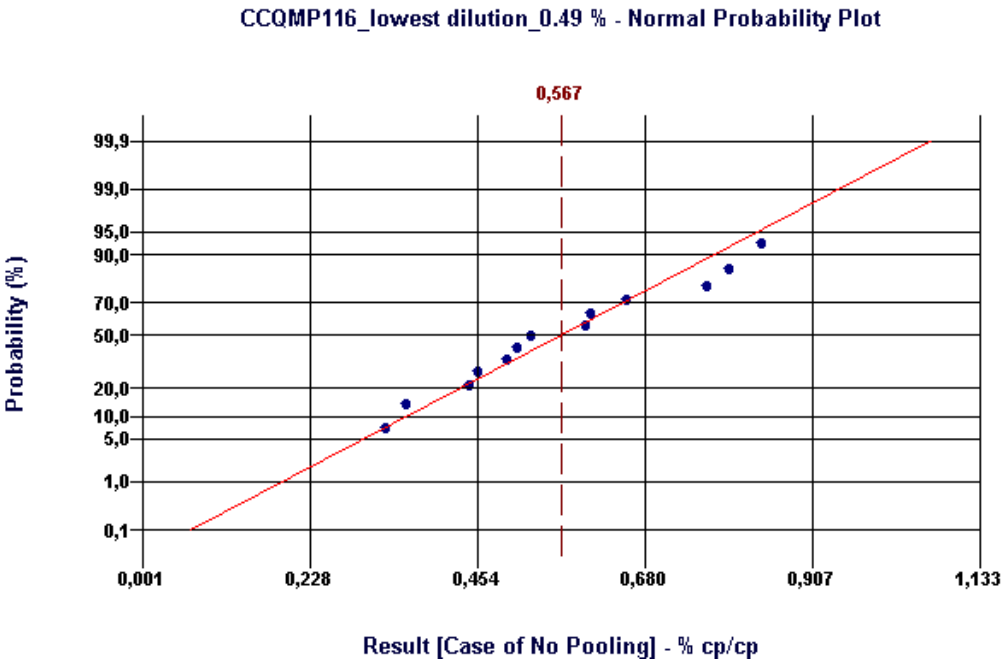
Mean of Lab ± StDev = 0,442 ± 0,029 [CV(%) = 6,6]

	Sample #2	Sample #4
Sample's ID =>		
Rep #1	0,455	0,396
Rep #2	0,458	0,441
Rep #3	0,473	0,426
Mean	0,462	0,421
STDev	0,010	0,023
CV(%)	2,087	5,442

Summary Table

Labs	Mean	U(k=2) [%]	STDev	Standard Error	H.W. CI (95%)	Sample #2	Sample #4
L1	0,608	0,0	0,052	0,037	0,470	0,645	0,571
L2	0,330	0,0	0,019	0,013	0,169	0,343	0,317
L3	0,655	0,0	0,006	0,004	0,055	0,651	0,660
L4	0,795	0,0	0,064	0,046	0,578	0,841	0,750
L5	0,837	0,0	0,203	0,143	1,821	0,980	0,693
L6	0,508	0,0	0,025	0,018	0,224	0,490	0,525
L7	0,454	0,0	0,015	0,011	0,136	0,465	0,444
L8	0,495	0,0	0,016	0,011	0,144	0,506	0,483
L9	0,527	0,0	0,005	0,003	0,042	0,523	0,530
L11	0,357	0,0	0,079	0,056	0,714	0,301	0,413
L12	0,765	0,0	0,177	0,125	1,588	0,890	0,640
L13	0,600	0,0	0,009	0,007	0,085	0,593	0,607
L14	0,442	0,0	0,029	0,021	0,260	0,462	0,421

	Case of No Pooling	Case of Pooling
# of Determinations	13	26
Range [min..max]	[0,330 .. 0,837]	[0,301 .. 0,980]
Average of Dataset	0,567	0,567
StDev of Average	0,162	0,169
R.S.D. of Average(%)	28,506	29,825
S.E. of Average	0,045	0,033
R.S.E. of Average(%)	7,906	5,849
95% H.W. Confidence Interval	0,098	0,068
95% H.W. Tolerance Interval	0,498	0,442



STATISTICAL ANALYSIS FOR CCQMP116_ 0.49 %

Labs	Method	Mean	STDev	H.W. CI (95%)	Samp#2	Samp#4
L0 -L1		0,608	0,052	0,470	0,645	0,571
L1 -L2		0,330	0,019	0,169	0,343	0,317
L2 -L3		0,655	0,006	0,055	0,651	0,660
L3 -L4		0,795	0,064	0,578	0,841	0,750
L4 -L5		0,837	0,203	1,821	0,980	0,693
L5 -L6		0,508	0,025	0,224	0,490	0,525
L6 -L7		0,454	0,015	0,136	0,465	0,444
L7 -L8		0,495	0,016	0,144	0,506	0,483
L8 -L9		0,527	0,005	0,042	0,523	0,530
L9 -L11		0,357	0,079	0,714	0,301	0,413
L10 -L12		0,765	0,177	1,588	0,890	0,640
L11 -L13		0,600	0,009	0,085	0,593	0,607
L12 -L14		0,442	0,029	0,260	0,462	0,421

meas. units : % cp/cp

Number of accepted Data sets (Labs) 13
Number of Individual Data (analysed samples) 26

Scheffe's multiple t-test
All Data Sets compatible two by two? No

TESTING FOR OUTLYING LAB MEANS

Dixon Test (a=0.05) Outliers NOT Detected
(a=0.01) Outliers NOT Detected

Nalimov t-test (a=0.05) Outliers NOT Detected
(a=0.01) Outliers NOT Detected

Grubbs test (Single) (a=0.05) Outliers NOT Detected
(a=0.01) Outliers NOT Detected

Grubbs test (Double)* (a=0.05) Outliers NOT Detected
(a=0.01) Outliers NOT Detected

* The Double Grubbs Test is valid only when the Single Grubbs Test
fails to detect any outlier

TESTING OF VARIANCES

Cochran Test
OutLying Lab variances? (a=0.05) No
(a=0.01) No

Bartlett test
Lab variances homogeneous? (a=0.05) Yes
(a=0.01) Yes

ANOVA

Between Labs StDev 0,261 % cp/cp
Within Labs (between samples) StDev 0,142 % cp/cp

Snedecor F-test
Differences between Labs statistically significant? (a=0.05) Yes
(a=0.01) Yes

Differences within Labs (between samples) statistically significant?(a=0.05) No
(a=0.01) No

Samples 1 and 3

Data from the Lab: L1

Mean of Lab ± StDev = 0,064 ± 0,024 [CV(%) = 37,8]

Sample's ID =>	Sample #1	Sample #3
Rep #1	0,061	0,040
Rep #2	0,089	0,038
Rep #3	0,094	0,063
Mean	0,081	0,047
STDev	0,018	0,014
CV(%)	21,868	29,558

Data from the Lab: L2

Mean of Lab ± StDev = 0,038 ± 0,003 [CV(%) = 7,4]

Sample's ID =>	Sample #1	Sample #3
Rep #1	0,050	0,020
Rep #2	0,040	0,048
Rep #3	0,030	0,040
Mean	0,040	0,036
STDev	0,010	0,014
CV(%)	25,000	40,062

Data from the Lab: L3

Mean of Lab ± StDev = 0,055 ± 0,002 [CV(%) = 3,0]

Sample's ID =>	Sample #1	Sample #3
Rep #1	0,054	0,031
Rep #2	0,062	0,068
Rep #3	0,053	0,063
Mean	0,056	0,054
STDev	0,005	0,020
CV(%)	8,757	37,176

Data from the Lab: L4

Mean of Lab ± StDev = 0,088 ± 0,003 [CV(%) = 3,7]

Sample's ID =>	Sample #1	Sample #3
Rep #1	0,072	0,085
Rep #2	0,120	0,078
Rep #3	0,080	0,095
Mean	0,091	0,086
STDev	0,026	0,009
CV(%)	28,364	9,935

Data from the Lab: L5

Mean of Lab ± StDev = 0,065 ± 0,021 [CV(%) = 32,9]

Sample's ID =>	Sample #1	Sample #3
Rep #1	0,07	0,02
Rep #2	0,071	0,05
Rep #3	0,1	0,08
Mean	0,080	0,050
STDev	0,017	0,030
CV(%)	21,211	60,000

Data from the Lab: L6

Mean of Lab ± StDev = 0,050 ± 0,004 [CV(%) = 7,0]

	Sample #1	Sample #2
Sample's ID =>		
Rep #1	0,068	0,047
Rep #2	0,042	0,053
Rep #3	0,048	0,043
Mean	0,053	0,048
STDev	0,014	0,005
CV(%)	25,849	10,559

Data from the Lab: L7

Mean of Lab ± StDev = 0,050 ± 0,002 [CV(%) = 3,8]

	Sample #1	Sample #3
Sample's ID =>		
Rep #1	0,049	0,057
Rep #2	0,039	0,039
Rep #3	0,058	0,058
Mean	0,049	0,051
STDev	0,010	0,011
CV(%)	19,530	20,830

Data from the Lab: L8

Mean of Lab ± StDev = 0,042 ± 0,002 [CV(%) = 4,0]

	Sample #1	Sample #3
Sample's ID =>		
Rep #1	0,037	0,033
Rep #2	0,052	0,048
Rep #3	0,039	0,040
Mean	0,043	0,040
STDev	0,008	0,008
CV(%)	19,089	18,609

Data from the Lab: L9

Mean of Lab ± StDev = 0,058 ± 0,017 [CV(%) = 28,8]

	Sample #1	Sample #3
Sample's ID =>		
Rep #1	0,080	0,047
Rep #2	0,050	0,050
Rep #3	0,080	0,042
Mean	0,070	0,046
STDev	0,017	0,004
CV(%)	24,744	8,723

Data from the Lab: L11

Mean of Lab ± StDev = 0,048 ± 0,010 [CV(%) = 21,5]

	Sample #1	Sample #3
Sample's ID =>		
Rep #1	0,059	0,055
Rep #2	0,034	0,080
Rep #3	0,030	0,032
Mean	0,041	0,056
STDev	0,016	0,024
CV(%)	38,332	43,126

Data from the Lab: L12

Mean of Lab ± StDev = 0,063 ± 0,009 [CV(%) = 14,9]

	Sample #1	Sample #3
Sample's ID =>		
Rep #1	0,050	0,060
Rep #2	0,050	0,080
Rep #3	0,070	0,070
Mean	0,057	0,070

STDev	0,012	0,010
CV(%)	20,377	14,286

Data from the Lab: L13

Mean of Lab ± StDev = 0,056 ± 0,020 [CV(%) = 36,4]

	Sample #1	Sample #3
Sample's ID =>		
Rep #1	0,050	0,11
Rep #2	0,021	0,042
Rep #3	0,053	0,058
Mean	0,041	0,070
STDev	0,018	0,036
CV(%)	42,757	50,790

Data from the Lab: L14

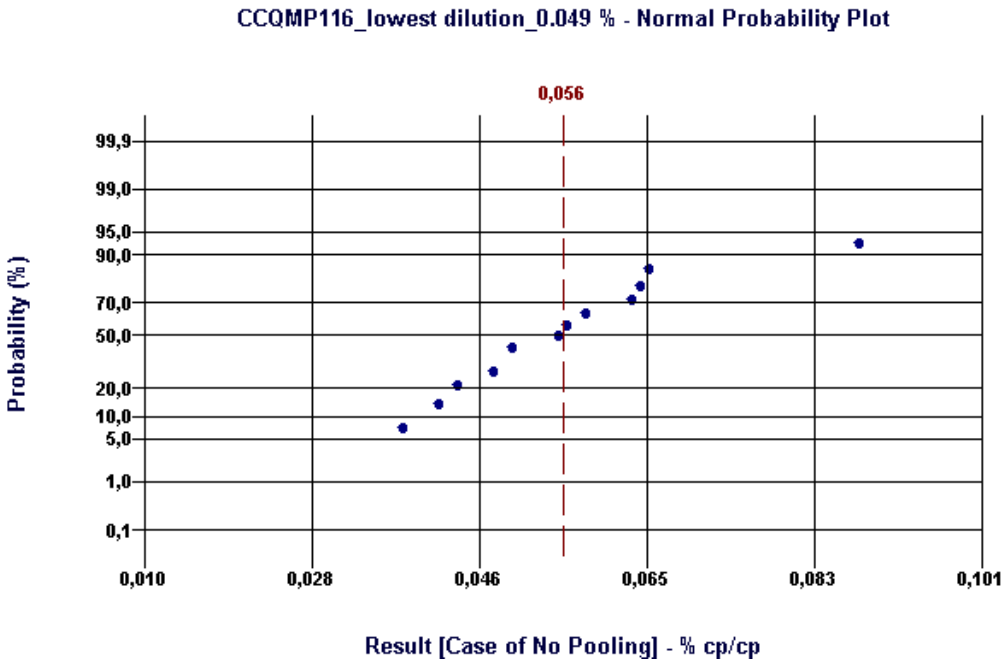
Mean of Lab ± StDev = 0,044 ± 0,002 [CV(%) = 5,4]

	Sample #1	Sample #3
Sample's ID =>		
Rep #1	0,037	0,063
Rep #2	0,043	0,027
Rep #3	0,057	0,037
Mean	0,046	0,042
STDev	0,010	0,019
CV(%)	22,474	43,897

Summary Table

Labs	Mean	U(k=2) [%]	STDev	Standard Error	H.W. CI (95%)	Sample #1	Sample #3
L1	0,064	0,0	0,024	0,017	0,218	0,081	0,047
L2	0,038	0,0	0,003	0,002	0,025	0,040	0,036
L3	0,055	0,0	0,002	0,001	0,015	0,056	0,054
L4	0,088	0,0	0,003	0,002	0,030	0,091	0,086
L5	0,065	0,0	0,021	0,015	0,193	0,080	0,050
L6	0,050	0,0	0,004	0,002	0,032	0,053	0,048
L7	0,050	0,0	0,002	0,001	0,017	0,049	0,051
L8	0,042	0,0	0,002	0,001	0,015	0,043	0,040
L9	0,058	0,0	0,017	0,012	0,150	0,070	0,046
L11	0,048	0,0	0,010	0,007	0,093	0,041	0,056
L12	0,063	0,0	0,009	0,007	0,085	0,057	0,070
L13	0,056	0,0	0,020	0,014	0,182	0,041	0,070
L14	0,044	0,0	0,002	0,002	0,021	0,046	0,042

	Case of No Pooling	Case of Pooling
# of Determinations	13	26
Range [min..max]	[0,038 .. 0,088]	[0,036 .. 0,091]
Average of Dataset	0,056	0,056
StDev of Average	0,013	0,016
R.S.D. of Average(%)	23,590	28,131
S.E. of Average	0,004	0,003
R.S.E. of Average(%)	6,543	5,517
95% H.W. Confidence Interval	0,008	0,006
95% H.W. Tolerance Interval	0,040	0,041



STATISTICAL ANALYSIS FOR CCQMP116_sample 1 and 3

Labs	Method	Mean	STDev	H.W. CI (95%)	Samp#1	Samp#3
L0 -L1		0,064	0,024	0,218	0,081	0,047
L1 -L2		0,038	0,003	0,025	0,040	0,036
L2 -L3		0,055	0,002	0,015	0,056	0,054
L3 -L4		0,088	0,003	0,030	0,091	0,086
L4 -L5		0,065	0,021	0,193	0,080	0,050
L5 -L6		0,050	0,004	0,032	0,053	0,048
L6 -L7		0,050	0,002	0,017	0,049	0,051
L7 -L8		0,042	0,002	0,015	0,043	0,040
L8 -L9		0,058	0,017	0,150	0,070	0,046
L9 -L11		0,048	0,010	0,093	0,041	0,056
L10 -L12		0,063	0,009	0,085	0,057	0,070
L11 -L13		0,056	0,020	0,182	0,041	0,070
L12 -L14		0,044	0,002	0,021	0,046	0,042

meas. units : % cp/cp

Number of accepted Data sets (Labs) 13
Number of Individual Data (analysed samples) 26

Scheffe's multiple t-test
All Data Sets compatible two by two? No

TESTING FOR OUTLYING LAB MEANS

Dixon Test (a=0.05) Outliers Detected
(a=0.01) Outliers NOT Detected
Lab L3 is an outlier at a=0.05

Nalimov t-test (a=0.05) Outliers Detected
(a=0.01) Outliers Detected
Lab L3 is an outlier at a=0.05
Lab L3 is an outlier at a=0.01

Grubbs test (Single) (a=0.05) Outliers Detected
(a=0.01) Outliers NOT Detected

Lab L3 is an outlier at a=0.05

Grubbs test (Double)* (a=0.05) Outliers Detected
(a=0.01) Outliers NOT Detected
Lab L3 is an outlier at a=0.05

* The Double Grubbs Test is valid only when the Single Grubbs Test fails to detect any outlier

---

TESTING OF VARIANCES

Cochran Test
OutLying Lab variances? (a=0.05) No
(a=0.01) No

Bartlett test
Lab variances homogeneous? (a=0.05)
(a=0.01) Yes

ANOVA

Between Labs StDev 0,017 % cp/cp
Within Labs (between samples) StDev 0,021 % cp/cp

Snedecor F-test
Differences between Labs statistically significant? (a=0.05) Yes
(a=0.01) Yes

Differences within Labs (between samples) statistically significant?(a=0.05) No
(a=0.01) No

19 Annex 5 : Uncertainty calculations reported by the participating laboratories

Lab 1

	Av. of GM/Ref (%)	SD(%)	UC form measurement, relative (%)	UC from each CRM, relative (%)	UC from callibrant , relative(%)
Day1					
Sample 1	0,080	0,017	21	-0.001;+0.080 1)	7 3)
Sample 2	0,81	0,026	3,2	-0.03;+0.04 2)	7 3)
Day2					
Sample 3	Under 5 copies				
Sample 4	0,62	0,032	5,2	-0.03;+0.04 2)	7 3)

For the select of GM/Ref value, I chose on the following points;
1. All measurement value (4) are acceptabe
2. Low template concentration is better than high conc for the inhibition.

References
1)http://www.irmm.jrc.be/html/reference_materials_catalogue/catalogue/attachements/ERM-BF418b_cert.pdf
2)http://www.irmm.jrc.be/html/reference_materials_catalogue/catalogue/attachements/ERM-BF418c_cert.pdf

Combined standard uncertainty (%)
- +
- +
- +

Lab 2

Measurement equation for real-time PCR:

$$T/E = f_{ext} \times f_{cal} \times f_{pcr} \times \frac{f_{dt} \times f_{st} \times D_{tr} \times 10^{\left(\frac{(y_{0t} - C_t)}{m_t}\right)}}{f_{de} \times f_{se} \times D_{er} \times 10^{\left(\frac{(y_{0e} - C_e)}{m_e}\right)}}$$

Definition of Symbols

E concentration of endogenous template in Unknown solution (copies/µL)

D_{er} dilution factor of Unknown used for real-time endogenous PCR

T concentration of transgene template in Unknown solution (copies/µL)

D_{tr} dilution factor of Unknown used for real-time transgene PCR

m_e and m_t slope of calibration curve for endogenous and transgene assays, respectively

C_e and C_t intercept of calibration curve for endogenous and transgene assays, respectively

Factors affecting uncertainty in result

f_{st} sampling error for transgene template

f_{dt} error in predicted response for transgene template

f_{se} sampling error for endogenous template

f_{de} error in predicted response for endogenous template

f_{ext} factor accounting for extraction uncertainty

f_{cal} calibrant uncertainty

f_{pcr} PCR uncertainty

Results

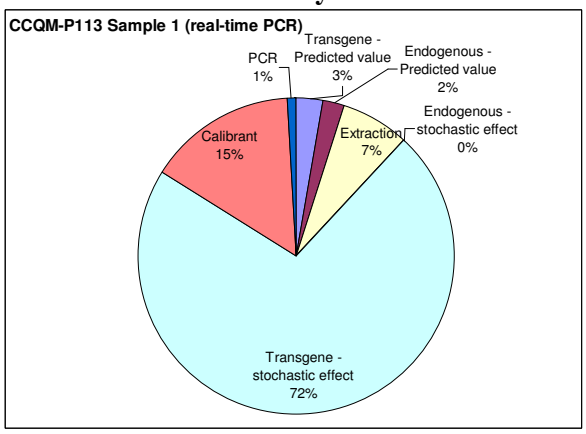
Measurand: copy number fraction of 1507 to hmg, expressed as a percentage, in the samples provided

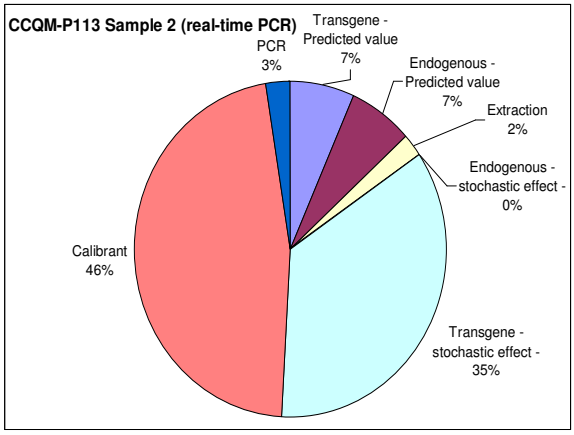
Units: percent copy number/copy number (% cp/cp)

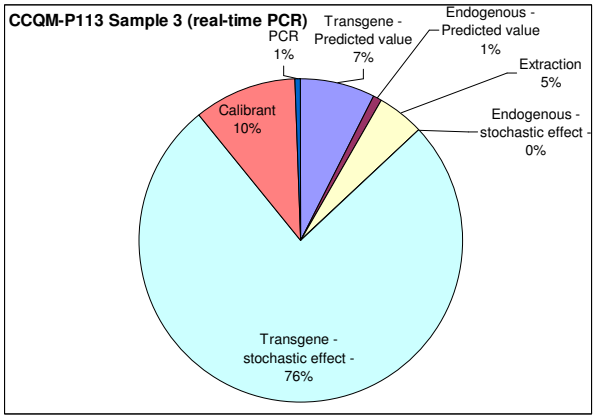
Coverage Factor (k): Coverage factor of 2 was used to determine the expanded uncertainty

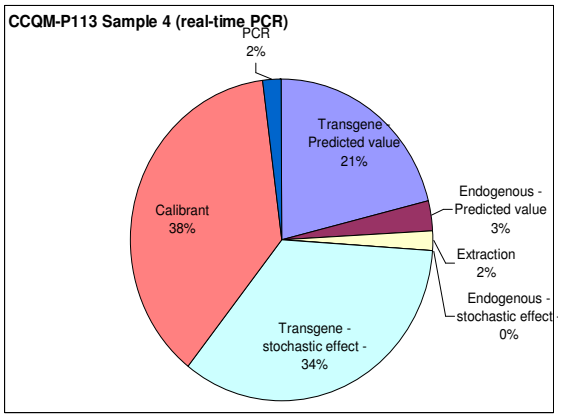
Sample 1	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Value Measured	**0.039 % cp/cp**	**0.342 % cp/cp**	**0.030 % cp/cp**	**0.321 % cp/cp**
Combined standard uncertainty	0.0069 % cp/cp	0.035 % cp/cp	0.0065 % cp/cp	0.037 % cp/cp
k (95%)	2	2	2	2
Expanded Uncertainty	**0.014 % cp/cp**	**0.070 % cp/cp**	**0.013 % cp/cp**	**0.073 % cp/cp**
Relative expanded uncertainty	35.8 %	20.5 %	43.8 %	22.9 %

Breakdown in uncertainty









Lab 3

The values and corresponding expanded uncertainties are:

Sample 1: 0.055 %GM ± 0.025 %GM
Sample 2: 0.67 %GM ± 0.17 %GM
Sample 3: 0.055 %GM ± 0.025 %GM
Sample 4: 0.66 %GM ± 0.17 %GM

Expanded uncertainties are quoted with coverage factors based on Students's t for the appropriate degrees of freedom.

The reported uncertainty is based on the dispersion of the observations in a single analytical run combined in quadrature with the uncertainty for the calibration material. This estimate makes no allowance for matrix effects, calibrant mismatch, long-term variation, extraction or other effects, and is consequently considered to represent an extreme lower bound on the uncertainty.

Lab 4

The result of Unknown sample and uncertainty (sent Tue 08/07/2008 10:35)

	Mean of all results	Combined Standard Uncertainty	Coverage factor	Expanded standard uncertainty (95% confidence leve)
Sample1	0.090506%	0.012%	2	0.024%
Sample2	0.840629%	0.011%	2	0.022%
Sample3	0.086116%	0.0099%	2	0.019%
Sample4	0.749674%	0.011%	2	0.022%

The result of Unknown sample and uncertainty (sent Mon 27/04/2009 03:34)

	Mean of all results	Combined Standard Uncertainty	Coverage factor	Expanded standard uncertainty (95% confidence leve)
Sample1	0.091%	0.18	2	0.032%
Sample2	0.84%	0.072	2	0.12%
Sample3	0.086%	0.092	2	0.016%
Sample4	0.75%	0.073	2	0.11%

Lab 5

	End (Avr. copy)	Ramdom Variation (RV, %)	GM (Avr. copy Nc	RV(%)	GM/END (C/C %)	Combined standard uncert
Day1						
Sample1	7500	1,6	4,9	25	0,065%	26
Sample2	7400	6,4	51	13	0,69%	16
Day2						
Sample3	11300	4,2	2,1	54	0,019%	55
Sample4	11200	2,4	85	15	0,76%	17

Uncertainty Budget	Standard uncertainty
Calibration solution	7
Pipetting (calibrant dilution)	1,414213562
Pipetting (sample dilution)	1,802775638
Pipetting (reaction mixture)	2,236067977
Ramdom Variation of Endogeneous gene (RV1)	
Ramdom Variation of GM gene (RV2)	
	59,25
UC of sample	

Lab 6

Results

Relative quantification of genomic DNA fragments extracted from a biological tissue	CCQM-P113

Result

Sample	Sample Code	Ratio 1507/hmg (%)*	Average cp ratio (%)	SD	Standard Uncertainty, u	Relative Uncertainty, U_{rel}	Combined Uncertainty	Expanded Uncertainty (%)
1	U1	0,068	0,052	0,0150	0,0050	0,0955	0,1184	0,0124
	U2	0,042						
	U3	0,048						
2	U4	0,436	0,49	0,1084	0,0361	0,0738	0,1017	0,0996
	U5	0,556						
	U6	0,478						
3	U7	0,047	0,048	0,0091	0,0030	0,0636	0,0946	0,0090
	U8	0,053						
	U9	0,043						
4	U10	0,621	0,52	0,1098	0,0366	0,0697	0,0988	0,1037
	U11	0,473						
	U12	0,482						

* The ratio is an average of the undiluted and all the diluted samples.

Methodology Outline

1 DNA extraction from samples 1, 2, 3 and 4. The CTAB method used for CCQM-P60 study was used.
2 DNA quantification of all DNA extractions with PicoGreen® dsDNA quantitation.
3 Determination of the copy ratio by real-time PCR. ABI TaqMan Universal 2-fold Buffer was used instead of the 10x buffer.
4 Result obtained from diluted samples were also included in the calculation of the average cp ratio, e.g. the ratio of sample U1 is the average value of U1, U1-2x and U1-4x.

Uncertainty Estimation

The Expanded Uncertainty is calculated in accordance witht the Guide to the Expression of Uncertainty in Measurement (GUM) with a coverage factor k = 2, corresponding to a level of confidence of about 95%.

Expanded Uncertainty	=	$ck\sqrt{U_{rel}^2+U_{CRM}^2}$
Standard Uncertainty, u	=	SD/√N
Relative uncertainty, U_{rel}	=	u /Mean ratio of the sample
Uncertainty of the calibrant, U_{CRM}	=	0.14/2 = 0,07
Combined Uncertainty	=	$\sqrt{U_{rel}^2+U_{CRM}^2}$
Coverage factor, k	=	2
Concentration, c	=	Mean ratio of the sample

Lab 7

The expanded measurement uncertainty U for a concentration c and a coverage factor k= 2 was estimated as taking into account the method repeatability, intermediate precision calculated for the 2 days and the uncertainty of the material.

$$U = c.k\sqrt{u_r^2 + u_{ip}^2 + u_m^2}$$

$$u_r = \sqrt{\frac{MS_{within}}{\overline{x}}}$$

$$u_{ip} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\overline{x}}$$

$$u_m = negligible$$

Day 1	Day 2
ratio 1507/hmg %	ratio 1507/hmg %
0,05	0,06
0,04	0,04
0,06	0,04

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Sample 1	3	0,146201514	0,048733838	8,81E-05
Sample 3	3	0,134791662	0,044930554	0,000116
			0,046832196	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2,16975E-05	1	2,16975E-05	0,212823	0,668534	7,708647
Within Groups	0,000407803	4	0,000101951			
Total	0,0004295	5				

repeatability **21,6%**

Method repeatability is the square of the within group MS divided by the average of the study

Between day variation **11,0%**

The between day to day variation is the square root of the difference of between groups and the within group variation divided by the number of replicates and divided by the average of the measurements

measurement uncertainty

					exp u	
uncertainty for	3	duplicates on	2	days	**14,7%**	**29,4%**

	uncertainty of the calibrant negligible	measurement uncertainty 0,0072	
		expanded U	rounded U
sample 1	0,05 ±	0,014	0,02
sample 3	0,04 ±	0,014	0,02
sample 1-3	0,05 ±	0,014	0,02

Day 1	Day 2
ratio 1507/hmg %	ratio 1507/hmg %
0,46	0,48
0,46	0,49
0,41	0,43

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Sample 2	3	1,330797735	0,443599245	0,001041
Sample 4	3	1,395147965	0,465049322	0,000746
			0,454324283	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0,000690159	1	0,000690159	0,772685	0,429024	7,708647
Within Groups	0,003572783	4	0,000893196			
Total	0,004262941	5				

repeatability **6,6%**

Method repeatability is the square of the within group MS divided by the average of the study

Between day variation **1,8%**

The between day to day variation is the square root of the difference of between groups and the within group variation divided by the number of replicates and divided by the average of the measurements

measurement uncertainty

					exp	
uncertainty for	3	duplicates on	2	days	**4,0%**	**8,0%**

	uncertainty of the calibrant	measurement uncertainty	
	negligible	0,018	
		expanded U	rounded
Sample 2	0,4 ±	0,04	0,1
Sample 4	0,5 ±	0,04	0,1
sample 2-4	0,5 ±	0,04	0,1

Lab 8

		Unknown 1		Unknown 2
	U1	0,04	U4	0,60
Day 1	U2	0,05	U5	0,48
	U3	0,04	U6	0,43
	U7	0,03	U10	0,51
Day 2	U8	0,05	U11	0,44
	U9	0,04	U12	0,49
Mean (%)		0,04		0,49
S.D		0,01		0,06
C.V(%)		17,16%		12,52%
IRMM Expanded Uncertainty(Ucr		0,014		0,14
IRMM Coverage factor; k (95%)		2		2
Std. Uncertainty (u) (SD/root n) n= 6		0,0076449		0,074423
u_rel (%) (std.U/mean x 100)		18,42		15,05
Degree of freedom		5,00		5,00
Coverage factor; k (95%)		2,57		2,57
Expanded Uncertainty(U) (std.U x k)		0,02		0,19
U-rel (%) (exp.U/mean x 100)		47,34%		38,68%

Lab 9

		average number of copies				
		1507	hmg	ratio 1507/hmg	U	Uexp
		(cp)	(cp)	% (cp/cp)	% (cp/cp)	% (cp/cp)
Sample 1	U1	15	9751	0,15	0,05	0,10
	U2	13	12186	0,11	0,04	0,08
	U3	16	10015	0,16	0,05	0,10
Sample 2	U4	115	10813	1,06	0,25	0,50
	U5	118	11046	1,07	0,25	0,51
	U6	110	10370	1,06	0,26	0,52
Sample 3	U7	12	12805	0,09	0,03	0,04
	U8	12	11974	0,10	0,04	0,04
	U9	11	13028	0,08	0,03	0,02
Sample 4	U10	116	11112	1,04	0,14	0,29
	U11	102	9104	1,12	0,20	0,41
	U12	109	11140	0,98	0,17	0,35

Lab 10

Sample 1 (U1-3, U7-9)

Relative cp target/cp reference = 0.21 ± 0.04

Source of uncertainty	Typical values	Standard uncertainty	Degree of freedom	Type of uncertainty
Regression from the calibration curve (reference) (copies number)	4.39	0.139	3	A
Regression from the calibration curve (target) (copies number)	3.7	0.219	3	A
Preparing calibration curve	1	0.07	large	B
Pipetting on to plats	1	0.004	large	B
Precision	1	0.049	5	A

u_c = 0.02

U = 0.04, k=2

Sample 1 (U4-6, U10-12)

Relative cp target/cp reference = 0.54 ± 0.28

u_c = 0.14

U = 0.28, k=2

Lab 11

	average number of copies		%				
	1507	hmg	ratio 1507/hmg		U%(K=2)		
U1	3	4690	0,06	0,0411	66	sample 1	0,027004
U2	2	5199	0,03				
U3	1	4308	0,03				
U4	15	4483	0,34	0,3010	22	sample 2	0,066367
U5	11	4292	0,25				
U6	12	4015	0,31				
	average number of copies		%				
	1507	hmg	ratio 1507/hmg		U%(K=2)		
U7	2,0	3629,04	0,06	0,06	51	sample 3	0,02857
U8	3,8	4768,11	0,08				
U9	1,9	5997,76	0,03				
U10	9,0	1599,00	0,56	0,41	32	sample 4	0,132089
U11	14,6	4728,71	0,31				
U12	13,5	3650,78	0,37				

Lab 12

Sample 1 (U1-U3)
% Relative cp target/cp reference = 0.22 $\pm$ 0.03 (k=2)
Sample 2 (U7-U9)
% Relative cp target/cp reference = 0.20 $\pm$ 0.02 (k=2)
Sample 3 (U4-U6)
% Relative cp target/cp reference = 0.34 $\pm$ 0.06 (k=2)
Sample 4 (U10-U12)
% Relative cp target/cp reference = 0.74 $\pm$ 0.13 (k=2)

Lab 13 (uncertainty calculation not yet received)

Lab 14

Uncertainty estimates values for P113. The estimates are based on standard error of the mean and the uncertainty of the calibrant supplied by IRMM.

Sample1	Sample 3			Combined 1,3	
0.037	0.063				
0.043	0.027				
0.057	0.037				
				Copy Fraction	**Relative (%)**
0.046	0.042		**Mean**	0.044	
0.010	0.019		**SD**	0.014	32
		u precision	**SE of the mean**	0.00559	13
		u calibrant	**Mean * 7%**	0.00307	7
			u combined	0.00638	15
		DF = 5, k=2.57	**U95**	0.016	37
Sample 2	**Sample 4**			**Combined 2,4**	
0.455	0.396				
0.459	0.441				
0.473	0.426				
				Copy Fraction	**Relative (%)**
0.462	0.421		**Mean**	0.442	
0.010	0.023		**SD**	0.028	6
		u precision	**SE of the mean**	0.01130	3
		u calibrant	**Mean * 7%**	0.03091	7
			u combined	0.03291	7
		DF = 5, k=2.57	**U95**	0.085	19

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Abstract
The provision of traceable standards to the biological community is an area of active research in many NMIs. The quantification of the relative amount of DNA sequences extracted from a biological tissue remains a complex analytical procedure and relies on the availability of such standards. Real-time PCR is currently the most applied measurement method to identify and quantify DNA sequences. Several NMIs were able to demonstrate their ability to use this technology to quantify a defined plasmid DNA using the same plasmid DNA as a calibrant (CCQM-P44 (1&2) and CCQM KC-61). The same measurement method was used to quantify genomic DNA extracted from a plant tissue and calibrated by a genomic DNA solution extracted from the same plant material (CCQM-P60). In a later study, the importance of a reliable DNA extraction method became apparent. The analytical procedure was more complex in CCQM-P60 compared to KC61, as it included a DNA extraction step. However, both studies were performed using matching calibrants for which a reference value had been assigned.
The goal of this pilot study was to demonstrate the ability to quantify DNA sequences present in a biological tissue using an independent calibration system. The quantification should ideally be performed by quantitative real-time PCR (QRT-PCR), but other methods not relying on the efficiency of thermal amplification such as digital PCR could also be applied.
The methodology requires extraction and purification of genomic DNA and accurate detection and quantification of the relative amount of two defined DNA sequences in the extracted genomic DNA.

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